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High-Performance Liquid Chromatographic Determination of $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] Ion Ratios in Seawater for Isotope Dilution Experiments

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A liquid chromatographic method with fluorometric detection, after postcolumn labeling with *o*-phthalaldehyde/2-mercaptoethanol reagent, was developed to directly quantify $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] ion ratios in aqueous samples that had been enriched with $^{15}NH_4$ for isotope dilution experiments. Cation-exchange chromatography, with a sodium borate buffer mobile phase, was selected as the separation mode because the two isotopes have slightly different constants in the equilibrium reaction between ammonium ion and ammonia. When the two forms of ammonium were passed separately through a high-performance cation-exchange column under precisely controlled chromatographic conditions, the retention time (RT) of $^{15}NH_4$ was 1.012 times the RT of $^{14}NH_4$. The two isotopic forms of ammonium ion were not resolved into separate peaks when they were injected together, but the retention time of the combined peak, as defined by an integrator, increased with increasing percentages of $^{15}NH_4$ in the mixture. The relationship of RT shift vs percentage of $^{15}NH_4$ relative to total ammonium followed a sigmoid-shaped curve with the maximum RT shifts per change in isotopic composition occurring between 25 and 75% $^{15}NH_4$. Using a calibration curve based on this relationship and a solution of separately injected $^{14}NH_4$ in mobile-phase buffer as an "internal standard", we were able to directly determine the concentrations and ratios of the two isotopes in enriched seawater.

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INTRODUCTION

Nitrogen recycling by organisms is a major process providing ammonium ion to primary producers in marine and freshwater food webs (*1–3*). Measurement of nitrogen recycling rates in aquatic systems is complicated by the fact that production and uptake processes occur simultaneously, often at similar rates, with the net result that ammonium ion concentrations do not necessarily change substantially even when recycling rates are relatively high (*4*). Isotope dilution experiments, involving the addition of [^{15}N]ammonium ion to water samples and the measurement of changes in the concentrations and isotopic composition of dissolved or particulate nitrogen in the sample over measured time intervals, provide a means to study nitrogen cycling rates in aquatic systems (e.g., refs 5 and 6). In these experiments, it is assumed that both ammonium isotopes will be taken up at about the same relative rates by organisms but that previously fixed nitrogen with a natural isotopic ratio ($^{14}NH_4$:[$^{15}NH_4$ + $^{14}NH_4$] = 0.9963) will account for most of the ammonium ion released by mineralization processes during short incubation intervals (hours). To calculate the ammonium ion regeneration and uptake rates by isotope dilution models (*5–7*), both ammonium ion concentrations and isotope ratios must be determined during short incubations.

The usual procedure to determine isotope ratios is to convert the fixed nitrogen to N_2 and then measure the nitrogen isotope ratios by mass or emission spectrometry. Conversion of particulate nitrogen to N_2 by combustion of dried samples in the presence of an oxidizing agent is relatively uncompli-

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cated (8), but preparation of the dissolved ammonium ion nitrogen for isotopic analysis is more demanding because ammonium ion must be quantitatively removed from solution and dried before the nitrogen can be converted to N_2. Several methods have been used to recover the dissolved ammonium ion from solution, including steam distillation (7), mercury precipitation (9), microdiffusion (4, 10), and incorporation into indophenol followed by solvent extraction (11) or isolation of the indophenol complex on a reversed-phase column (12). Each water sample must be large enough (e.g., 50–500 mL) to provide enough nitrogen (>100 ng of atom N sample^{-1}) for mass analysis. Also, care must be taken during sample preparation to prevent nitrogen contamination from reagents, glassware, or atmospheric sources. Nitrogen cycling rates usually cannot be determined during shipboard incubations because nitrogen from the processed samples must be returned to a land-based laboratory for isotope analysis. A relatively large number of time-course samples must therefore be processed in the field to assure that some of the selected incubation intervals will be long enough to show significant changes but short enough to prevent extensive recycling of the ^{15}N label (7, 11).

Isotope dilution experiments would be more convenient if concentrations and nitrogen isotope ratios of the dissolved ammonium ion could be determined directly on relatively small samples during the course of field or laboratory incubations. Analysis of the isotopes by aqueous high-performance liquid chromatography (HPLC) is potentially feasible because the two isotopes have different constants for the equilibrium between ammonium ion and ammonia in aqueous systems. At equilibrium, a slightly higher percentage of ^{15}N than ^{14}N exists in the protonated form relative to the fraction in the nonionic form (13, 14). Nitrogen isotopes have been purified by ion exchange (e.g., refs 15–17), but this approach has not been adapted to the analytical determination of isotope ratios. Nitrogen and oxygen isotopes of small polar organic compounds have recently been separated by reversed-phase HPLC, but elution times of about 10 h were required for effective separations (18). The current availability of high-performance cation-exchange columns and pumps combined with sensitive fluorometric detection techniques for ammonium (19–21) allows direct determination of $^{14}NH_4$:[$^{15}NH_4$ + $^{15}NH_4$] ion ratios by HPLC in solutions containing comparable concentrations of the two isotopic forms of ammonium. In this paper, we describe and evaluate an HPLC method to simultaneously measure the concentration and nitrogen isotope composition of ammonium in seawater that had been enriched with $^{15}NH_4$ for isotope-dilution experiments.

EXPERIMENTAL SECTION

The HPLC system consisted of an Anspec 909 (now available as an Alcott Model 760) pump, with a microbore head, or a high-performance syringe pump (ISCO SFC-500 or 260D Syringe Pump), a Rheodyne 7125 sample injection valve with a 50-μL sample injection loop, a heated (47 °C; Standard CROCO-CIL HPLC column heater) 30-cm × 4-mm-i.d. stainless steel column containing a strong cation-exchange resin (5-μm beads of sodium-form sulfonic acid cation exchanger with 12% cross-linked polystyrene/divinylbenzene polymeric matrix; St. John Associates), a postcolumn o-phthalaldehyde (OPA)/2-mercaptoethanol reaction system [consisting of a reagent reservoir, a reagent delivery pump (Rainin or Technicon peristaltic pump) and a heated (47 °C) reaction coil (a Teflon tube, 0.2-mm i.d. × ca. 1 m, interwoven around three metal prongs 3-mm apart to assure thorough mixing)], and a fluorometric detector (Gilson Model 121). The excitation and emission light filters were a Corning 7-60 (maximum transmission at 356 nm) and a Corning 3-71 (sharp cutoff at 482), respectively. A Shimadzu C-R3A integrator, with the peak width set at 3 s, recorded the areas and retention times of the chromatographic peaks. A second integrator (Hewlett-Packard 3394A) was connected to the fluorometer in parallel with the Shimatzu integrator to help estimate how much of the observed chromatographic variability in estimating retention time shifts was due to integrator error.

The isocratic elution buffer used to evaluate the method was prepared by adding 12.0 g of boric acid, 12.0 g of NaCl, and 0.8 g of ethylenediaminetetraacetic acid (EDTA, disodium form) to about 990 mL of distilled and deionized water (DDW), adjusting the pH to 10.30 (Mallinckrodt pH 10.00 calibration buffer) with NaOH solution (final volume 1 L) and passing the solution through a DDW-rinsed 0.22-μm pore size nylon filter. Other combinations of boric acid and sodium chloride adjusted to different pH's were also tried to examine the effects of buffer composition on the retention times and separation characteristics of the two isotopic forms of ammonium. During buffer preparation, care was taken to prevent human exposure to the caustic NaOH solution. The buffer was kept in a filled, closed glass container or under an inert gas (e.g., Ar) to prevent atmospheric carbon dioxide from affecting the buffer pH during storage.

The OPA/2-mercaptoethanol reagent resembled that of Hare (22) except that the pH was adjusted to 7.0 instead of 9.5 to optimize the fluorometric response of OPA–ammonium relative to that for OPA–amino acid derivatives (23, 24). Fifteen grams of boric acid was added to about 495 mL of DDW, and the pH was adjusted to 7.0 with KOH pellets. In a separate container, 0.25 g of specially purified OPA (Sigma No. PO657) was dissolved in 5 mL of ethanol and 0.25 mL of 2-mercaptoethanol. This solution was then added to the pH 7 borate solution. Direct human contact to KOH, OPA, or 2-mercaptoethanol was avoided during reagent preparation. The 2-mercaptoethanol was handled under a hood to prevent vapor inhalation. The reagent solution was stable for at least 7 days at room temperature.

Primary standard solutions of 1 mM $^{14}NH_4$ (99.63% $^{14}NH_4$) and 1 mM $^{15}NH_4$ (98% $^{15}NH_4$, Sigma) were prepared in DDW and stored at room temperature. These primary standards were used for water additions and standard preparations.

For method evaluation, the buffer was pumped through the column at a flow rate of about 0.15 mL min^{-1} to give an $^{14}NH_4$ retention time (RT) of about 36 min. Because the RT's at a given flow rate somtimes varied slightly with different preparations of mobile-phase buffer, the buffer flow rate was adjusted after preparation of a new buffer, if necessary, to assure that the RT for the $^{14}NH_4$ in buffer would remain constant (±0.4 min) while a given calibration curve was being used. Reagent solution was pumped into the postcolumn mixing coil at a rate of 0.10 mL min^{-1}. For analysis, about 0.4 mL of water sample was passed directly through a 0.22-μm pore size filter (clamped in a St. John No. A5–5201 filter assembly) directly through the 50-μL sample loop to rinse the filter and injection system and load a clean sample of filtrate into the loop for HPLC analysis.

For each sample run, the following solutions were sequentially filtered and injected at precise intervals: at 0 min, 2 μM standard $^{14}NH_4$ solution prepared in mobile-phase buffer; at 3.00 min, 2 μM standard $^{14}NH_4$ solution prepared in mobile-phase buffer; at 6.00 (or 8.00) min, sample filtrate.

Sample ammonium ion concentrations were determined by comparing the sample ammonium ion peak areas to the mean areas for the ammonium ion peaks from the standard solutions that were injected at 0 and 3 min. Fluorescence response is linear with concentration for OPA derivatives of ammonium over a wide concentration range (e.g., 0–40 μM; ref 24). Peak area comparisons were more appropriate than peak height comparisons for quantifying total ammonium ion concentrations of the isotope mixtures because peak widths and heights were affected by the isotopic composition of ammonium in the samples; mixtures of the isotopes produced wider and shorter peaks than did either isotope in pure form. To accurately quantify ammonium concentrations in seawater samples, it was necessary to correct for "hidden ammonium" (about 0.3 μM) in a small baselin trough, caused by the seawater matrix, that occurred under the sample peak but not under the standard peaks. Ammonium in the trough was obscured from integrator detection in fortified samples because the small negative peak had about the same retention time as ammonium.

The isotopic composition of ammonium ion was determined from a sigmoid-shaped calibration curve obtained by nonlinear regression of the RT shift ($RT_{samp}(NH_4)$ – RT($^{14}NH_4$ in buffer))

against the ion ratio of $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] in isotope mixtures prepared in solutions having the same chemical matrix (e.g., seawater) as the samples (see Results and Discussion section). The mobile-phase buffer was used as the solvent for the $^{14}NH_4$ calibration solution to prevent the sample ammonium ion RT from being affected by the matrix of the standard solution. The RT of the $^{14}NH_4$ standard in mobile-phase buffer that had been injected exactly 3.00 (or 5.00) min before the sample was subtracted from the RT of the sample ammonium ion peak to obtain the RT shift. This "internal" comparison compensated for slight chromatographic variations among repetitive HPLC runs.

A series of standard seawater mixtures containing 2 μmol of added ammonium ion with various ratios of $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] were analyzed to define the shape and precision of the calibration curve. The standards used for this curve were analyzed over a period of 4 days so that the points would include day-to-day, as well as within-day, variations. The standards analyzed on each respective day were distributed over the range of isotope ratios to minimize day-to-day bias in the shape of the composite curve. Day-to-day stability of the calibration curve was important because time constraints normally would prohibit the analysis of a sufficient number of standards to define a detailed calibration curve on the same day that experimental samples are analyzed. Although statistically significant ($p < 0.10$), the day-to-day variance constituted only about 30% of the total variance.

The ability of the HPLC technique to determine isotope ratios was evaluated by spiking a second series of seawater samples with different ratios of $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] and estimating their apparent isotopic composition from the initial calibration curve. The measured isotopic ratios were then compared to the actual ratios to help define the reliability and precision of the method in determining isotope fractions for unknown samples.

To compare isotope ratio results obtained by HPLC with those obtained with emission spectroscopy, the HPLC system was transported to the Academy of Natural Sciences of Philadelphia. Three 100-mL samples of low-ammonium seawater were separately spiked with 2 μM ammonium ion consisting of different combinations of $^{1}15NH_4$ and $^{14}NH_4$ to give isotope ratios within the specified range of 25–75% $^{15}NH_4$. Two sets of subsamples, each consisting of three 9-mL portions from each of the three spiked samples, were removed and filtered (Rainin nylon syringe filter; pore size 0.2 μm). The first 3 mL of each filtrate was discarded and the remainder was placed in a clean glass vial. A similar set of nine subsamples was provided, in random order, to each analyst. Information on the exact isotope composition was not provided to the analysts until after the freshly prepared samples were analyzed. The HPLC samples were analyzed directly for $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] ratios as described above except that the buffer was pumped with an ISCO 260D syringe pump and the OPA reagent was delivered with the microbore piston pump equipped with an UpChurch P-738 back-pressure device. Also, sample injections were made at 5.00 min, rather than 3.00 min, after injection of the second standard solution, and a new calibration curve was prepared. Portions of the sample filtrates provided for HPLC analysis were frozen for 2 days and then thawed and analyzed again to examine possible effects of freezing on isotope ratio determinations.

The emission spectroscopy subsamples were each fortified with 2 μM $^{14}NH_4$ in DDW to reduce the isotopic ratio of the sample to the range normally analyzed by emission spectroscopy (0.4–5% $^{15}NH_4$). The ammonia was converted to indophenol by using a modification of the phenol–hypochlorite reaction for ammonium analysis (*11*). After the pH was adjusted to 6.3, the indophenol was extracted on octadecyl (C_{18}) columns (Baker-10SPE), eluted with 2 mL of HPLC-grade acetone, concentrated by partial evaporation, and dried onto precombusted glass fiber filters (Whatman GF/F) (*25*). Samples were converted to nitrogen gas by a modified Dumas combustion procedure (*26*) after they were sealed into quartz tubes under vacuum. They were analyzed for isotopic composition by emission spectroscopy using previously described procedures (*27*).

RESULTS AND DISCUSSION

In developing a fractionation technique, we wished to maximize the ratio of isotope separation:chromatographic RT, while keeping the total analysis time limited to about 45 min.

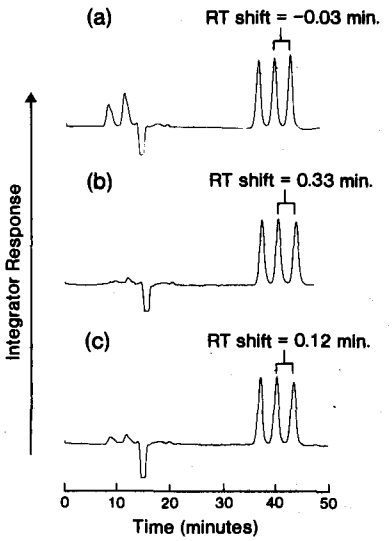

Figure 1. Chromatograms for seawater containing 2.2 μM NH_4 with the following ion ratios of $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$]: (a) 0.0037 (i.e., natural abundance), (b) 0.73, (c) 0.46. The first two ammonium peaks on each chromatogram were ammonium standards prepared in mobile-phase buffer. The third peak was for ammonium ion in spiked seawater containing a mixture of the two isotopes. The RT shift was calculated by subtracting the RT of ammonium ion peak 3 from that of ammonium ion peak 2 in each chromatogram.

A 12% cross-linked resin with small (5-μm-diameter) resin particle size was chosen to optimize separation while maintaining high column efficiency at low flow rates. The separation factor (i.e., difference in RT for two peaks) of cation-exchange columns increases with increased cross-linkage of the resins at low buffer flow rates (*28*).

Various combinations of sodium chloride, boric acid, and pH and different chromatographic conditions were evaluated in an attempt to obtain separation of the two isotopic forms of ammonium. The RT's were consistently longer for $^{15}NH_4$ than for $^{14}NH_4$ for the buffers that we examined (pH's between 9.7 and 10.3). This observation is consistent with the idea that the RT's relate to the respective constants for the equilibrium relationships between ammonium ion and ammonia. Ammonium ion retention times were too long to be practical for routine analysis at buffer pH's near the pK of about 8.6 for the dissociation reaction between ammonium ion and unprotonated ammonia at 47 °C (*29*). For example, a buffer, having the same chemical composition as our described buffer, but adjusted to pH 9.73 with sodium hydroxide, produced RT's of 80.7 and 81.8 min for $^{14}NH_4$ and $^{15}NH_4$, respectively, at buffer flow rates of ca. 0.15 mL min^{-1}. Flow rates were generally kept under 0.18 mL min^{-1} to keep the column pressure below 24 mPa, the approximate pressure where the column resin beads may begin to deform. Retention times could be decreased either by increasing the ionic strength of the sodium chloride and/or by increasing the pH of the buffer, but either option caused some reduction in separation efficiency for the two isotopes. The described buffer was selected for method evaluation because it produced an ammonium retention time of about 36 min and produced a symmetrical calibration curve with measurable slopes on each end of the curve. Also, it tended to center the ammonium peak over the small seawater matrix baseline trough and thereby minimized any effects on RT shifts that could potentially result from the ammonium peak eluting over a shoulder of the trough.

When concentrations of total ammonium ion were 2 μM or greater, the RT shift for $^{15}NH_4$ was about 0.4 min longer than that for $^{14}NH_4$ (Figures 1 and 2). At very low concentrations (e.g., 0.2 μM in our unspiked test seawater), the RT shift of $^{14}NH_4$ was strongly skewed as compared to a constant

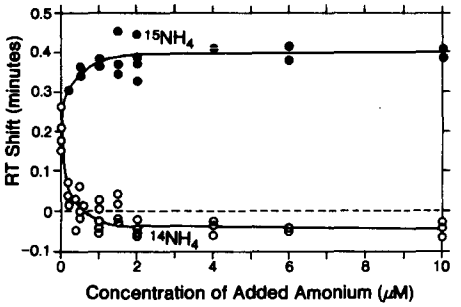

Figure 2. Relationship of ammonium ion concentration to retention time shift, relative to the retention time of 2 μM $^{14}NH_4$ in mobile-phase buffer, for $^{14}NH_4$ and $^{15}NH_4$ in seawater. The natural ammonium concentration (i.e., the concentration at zero ammonium additions) was 0.2 μM.

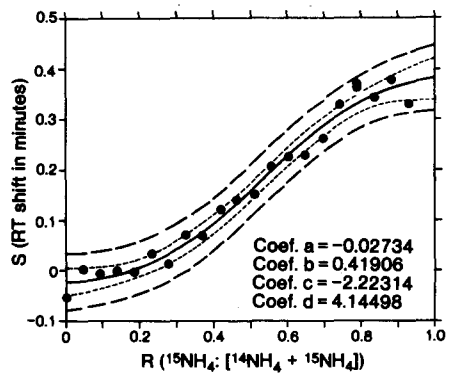

Figure 3. Calibration curve showing retention time shift, relative to that for 99.63% $^{14}NH_4$ in mobile-phase buffer, vs the percentage of total ammonium ion present as $^{15}NH_4$.

shift for concentrations above 2 μM (Figure 2). Moderate skewing was also apparent at concentrations between 0.5 and 2 μM. The direction of skewing at low concentrations appeared to be related to the chemical matrix of the samples. For example, in freshwater samples, $^{14}NH_4$ RT's decreased at very low concentrations (data not shown), whereas in seawater the RT's increased (Figure 2). Because the RT shifts were skewed at low ammonium concentrations, isotope ratios could not be accurately examined for seawater samples containing total (natural plus added) ammonium concentrations of less than about 1–2 μM. Therefore, we developed and evaluated the HPLC method using 2 μM ammonium ion additions with different ratios of the two isotopes. Although not investigated here, lower concentrations of $^{14}NH_4$ than 1 μM could potentially be examined by isolating the ammonium from relatively large samples with a concentrator column. The analytical column itself could likely serve as an efficient concentrator column if the pH's of the samples were reduced and the sample loop volume was increased.

Although the two isotopic forms of ammonium could not be resolved into separate peaks when injected as mixtures, the integrator-determined RT's of the combined peaks increased with increasing fractions of $^{15}NH_4$ in the mixtures (Figure 3). Retention time shifts of ammonium ion from samples, relative to that of $^{14}NH_4$ in the standard buffer solutions, were chosen over other peak characteristics for measurement because they followed predictable patterns with changes in isotope ratios and were automatically defined by the integrator in an unbiased manner.

When using RT shifts of the combined peak to identify isotope ratios in seawater samples, it was necessary to reduce other factors causing RT variability so that isotopic shifts could be differentiated and quantified. To minimize RT variability, we used high-precision pumps to deliver both the mobile-phase buffer and reagent and maintained the column at a constant temperature (47.0 ± 0.1 °C). When using the microbore piston pump for the buffer, we monitored and recorded system back-pressure during chromatographic runs, by connecting a recorder to a "pressure output" port on the pump, to verify that the buffer flow rates remained constant during and among chromatographic runs. Other than normal short-term pump-cycle pressure changes, the pressure usually remained constant at about 20 mPa except for brief injection signals and a slight pressure depression caused by sea salts after seawater was injected. Other occasional moderate pressure fluctuations within runs, presumably caused by slight check-valve leakage, did not measurably affect isotope ratio results if they occurred after the last sequential injection but before the first peak was eluted, but they caused significant errors if they occurred between sequential injections or during the elution of the ammonium peaks. If pressure fluctuations were observed during these critical periods, the chromatographic data were not used for isotope ratio determinations. The above precautions were not necessary for the syringe pumps that produced constant, pulseless flow rates.

The remaining chromatographic variations in measured RT's among or within runs were compensated for by using the "sequential injection" technique to obtain relative RT shifts for the different isotope combinations. Subtracting the RT observed for sample ammonium from that for the adjacent standard solution provided an internal calibration for RT shifts. The ammonium solution in buffer was also used as the standard to quantify the concentration of total ammonium in the injected sample. Thus, both the ratio, R, of $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] and the total pool size, P, of ammonium [i.e., the two measurements needed for the Blackburn (*5*) or Caperon et al. (*6*) models to calculate ammonium uptake and regeneration rates] could be obtained from the same chromatograms at given time points during an isotope dilution experiment.

Ammonium ion peak RT shifts followed a sigmoid pattern with change in the isotopic composition in seawater (Figure 3). The sample RT shifts were negative for low fractions of $^{15}NH_4$ because the RT's for $^{14}NH_4$ in seawater were slightly shorter than the RT's for $^{14}NH_4$ in the mobile-phase buffer. The sigmoid relationship of RT shift, S, to the ratio, R, of $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] was modeled as the renormalized cumulative normal distribution function:

$$S = a + b\Phi(c + dR) + \epsilon$$

where a, b, c and d are parameters to be fitted, $\Phi(\cdot)$ is the cumulative normal distribution function, and ϵ is a random error term. This response function fits the data quite well over the complete domain of $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] ratios (Figure 3). The residuals of the fitted data were modeled as the sum of a random-effect variable for day of analysis and an "overall" random variable for variation within days (see ref 30). Nonlinear least-squares fitted parameter values were $\hat{a} = -0.027\,34$, $\hat{b} = 0.419\,06$, $\hat{c} = -2.2231$, and $\hat{d} = 4.145$, with a residual mean square error of 0.000 5241 ($df_{error} = 18$). Curves, delimiting 95% confidence intervals for a predicted response (S) to a given value or R, were computed following the methods of Bates and Watts (*31*) modified to address a predicted response. These (upper and lower) confidence curves along with the predicted response curve (Figure 3) were used as in Draper and Smith (*32*) to reverse-predict R values with upper and lower 95% confidence intervals (e.g., Figure 4). The inner dotted lines on Figure 3 delimit 95% confidence intervals for the mean response (fitted solid line) at given R values.

The exact shape of the calibration curves depended on the chemical composition and pH of the mobile-phase buffer, but the largest peak shifts relative to unit changes in isotopic composition were observed in the region between 25 and 75% $^{15}NH_4$. To take advantage of the region of maximum RT shifts

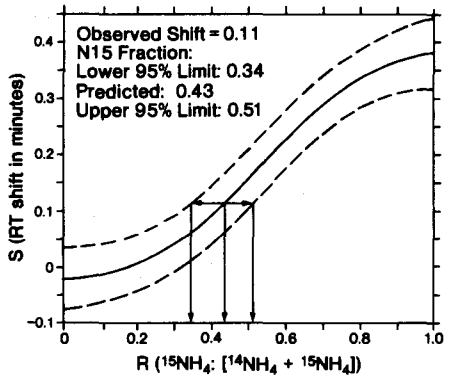

Figure 4. Schematic example of reverse prediction, with 95% confidence intervals, of $^{15}NH_4$ isotope ratio (R) from a measured RT shift (S) of 0.114 min.

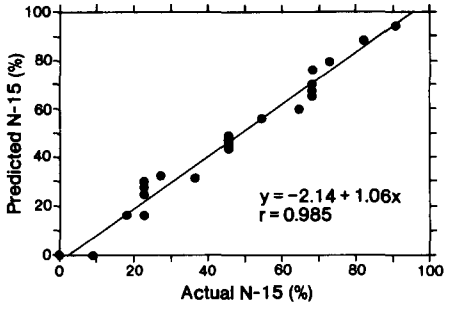

Figure 5. Comparison of predicted vs actual isotope ratios. Predicted ratios were determined by HPLC using a calibration curve (Figure 3) obtained earlier from another set of spiked samples. Actual ratios were based on the amounts of the two isotopes added and the natural level of ammonium ion in the seawater. The sum of added $^{14}NH_4$ and $^{15}NH_4$ was 2 μM for each addition; the natural ammonium ion concentration was 0.19 μM in the seawater.

Table I. Mean Retention Time Shifts[a] and Standard Deviations (N = 5) for Samples Containing Ratios of 0.23, 0.45, and 0.67 $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] in a Total Ammonium Ion Concentration of 2.19 μM in Seawater[b]

isotope comp	mean RT shift, min	SD, min
0.23	0.019	0.020
0.45	0.136	0.017
0.67	0.282	0.023

[a] Relative to $^{14}NH_4$ in mobile-phase buffer. [b] I.e., 2 μM ammonium ion containing 25, 50, or 75% $^{15}NH_4$ was added to seawater having a natural ammonium ion concentration of 0.19 μM.

per change in isotopic composition, isotope dilution experiments should preferably be designed to maintain R values between 0.25 and 0.75 during the course of the experiments. Although appropriate ranges of ammonium ion concentrations and isotope ratios can be maintained by experimental manipulation, the need to keep ammonium concentrations at > 1 μM and R at > 0.25 precludes the method as described from being used for truly "tracer-level" experiments (i.e., $^{15}NH_4$-additions of less than 15% of ambient ammonium ion concentrations; ref 33). The addition of more than trace amounts of ammonium ion can affect ammonium ion uptake rates and, to some extent, nitrogen regeneration rates as a result of increased phytoplankton growth (*34*). The results obtained in isotope dilution experiments with more than tracer additions of $^{15}NH_4$ therefore may represent "potential" rates more accurately than they do "actual" rates. Further refinement of the HPLC technique to resolve the two isotopic forms of ammonium into separate peaks could potentially extend the capability of this approach for use in experiments with tracer level additions of $^{15}NH_4$.

Replicate measurements of respective samples containing isotope ratios of 0.23, 0.45, and 0.67 yielded mean RT shifts and standard deviations (SD) (Table I) similar to those predicted by the calibration curve (Figure 3). The precision of the method for RT shifts was approximately SD = 0.02 min at each of these concentrations; similarly, the standard deviation for RT differences of sequentially injected standards was 0.02 min. A RT shift standard deviation of 0.02 min represents a methodological precision of < ±0.05 for R in the region of the calibration curve between 0.25 and 0.75 $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] (Figure 3). The accuracy of the method for experimental measurements can of course be improved by making multiple injections and averaging the results or by making time series measurements during an experiment.

A part of the observed variability in RT shifts can be attributed to integrator variability in determining retention times. The integrator variability is potentially greater for RT shift determinations than it would be for single peaks because the RT shifts are determined as differences between two chromatographic peaks. When two integrators were connected in parallel to monitor the same fluorometric signal, the mean absolute difference in RT shifts obtained from the two integrators was 0.009 min (SD = 0.007, N = 19). However, the direction of RT shifts was unbiased between the two integrators as the mean RT shifts obtained from them were not statistically different. We now routinely compare results from two integrators to detect occasional discrepencies caused by integrator error in determining accurate RT's.

The described HPLC method for ammonium ion concentration and isotope ratio determinations was generally free of interferences from other compounds in seawater (e.g., Figure 1). Low-quality OPA (97% purity) produced some unidentified peaks from seawater that were not observed with the specially purified OPA (Sigma No. PO657). The OPA/2-mercaptoethanol reagent forms fluorescent derivatives with primary amines such as amino acids, but most of these compounds were eluted before ammonium ion under the conditions described here. Added arginine co-eluted with ammonium ion at 47 °C but could be resolved from ammonium ion by lowering the column temperature to 38 °C. Unfortunately, the calibration curve was not as symmetrical when run at 38 °C as it was at 47 °C. Arginine does not normally interfere with ammonium ion analyses because it is rarely if ever detected in unfortified seawater. However, in situations where arginine may be present (e.g., if amino acids are added to incubation vessels as in substrate-addition experiments), it would be necessary to modify conditions so that arginine would be separated from ammonium.

To evalute the ability of the HPLC RT-shift technique to determine isotope ratios in seawater from the calibration curve of Figure 3, seawater samples were spiked with 2 μM ammonium ion having various ion ratios of $^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$] and then analyzed by HPLC. The R values were reverse-predicted from RT shifts using the calibration relationship, obtained with the pH 10.3 buffer (Figures 3 and 4), and compared to the isotopic ratios calculated from the actual amounts added (Figure 5). Although the analyses were done several weeks after the initial calibration curve was prepared and different pumps were used for mobile phase delivery (syringe pump for these samples vs microbore piston pump for the calibration curve), the retention times were similar (about 36.5 min). A highly significant (r = 0.985) linear relationship, with a slope of 1.06, was observed between predicted and actual values (Figure 5). This result indicates that the relationship between RT shifts and isotopic composition is robust if buffer composition and other chromatographic conditions remain constant, as indicated by consistent retention times.

Table II. Comparison of Isotope Ratio ($^{15}NH_4$:[$^{14}NH_4$ + $^{15}NH_4$]) Results Obtained from Seawater[a]

calcd isotope ratios	predicted isotope ratios: HPLC fresh $\bar{X}$	HPLC fresh SD	HPLC thawed $\bar{X}$	HPLC thawed SD	emission spectroscopy $\bar{X}$	emission spectroscopy SD
0.27	0.29	0.03	0.32	0.01	0.17 (0.35)	0.01
0.46	0.47	0.03	0.45	0.05	0.27 (0.45)	0.01
0.65	0.59	0.02	0.61	0.03	0.41 (0.59)	0.04

[a] Seawater originally contained 0.16 μM NH_4 and was spiked with 2 μM combinations of $^{14}NH_4$ + $^{15}NH_4$ to give final ratios of 0.27, 0.46, and 0.65. Subsamples were immediately filtered (0.2-μm pore size), randomized, and provided for HPLC and emission spectroscopy analysis. Filtrates for HPLC analysis were analyzed fresh, but second portions were also analyzed after the filtrates were frozen for 2 days. Emission spectroscopy samples (3.5 mL) were diluted with 196.5 mL of 2 μM NH_4 (natural isotope abundance) in distilled water so they would fall within the range of enrichment required for emission spectroscopy. The emission spectroscopy values were not initially corrected for possible ^{14}N contamination during sample preparation. When these numbers were arbitrarily corrected (values in parentheses), by adding the overall mean difference (0.18) between the calculated and predicted values to the experimental mean values, values were obtained that would be comparable to those observed in field experiments where experimental results are corrected based on mean recovery efficiency for spiked samples.

Analysis of the randomized subsamples by HPLC, in the experiment comparing the HPLC method with emission spectroscopy, provided estimates of the respective isotopic ratios and accurately predicted which subsamples were replicates of each other. The mean values measured by HPLC were within 10% of the values that were calculated from the isotopic composition of both the natural and added ammonium (Table II). Isotope ratio results from the frozen filtrates were not significantly different from those obtained on the freshly spiked samples. The emission spectroscopy method also grouped the replicate subsamples accurately, but the values obtained by emission spectroscopy were consistently lower than the calculated ratios (Table II). We believe that this discrepency was likely caused in part by dilution of the sample $^{15}NH_4$ by ^{14}N that was introduced as contamination during the ammonium extraction and/or sample preparation steps. Separate samples spiked with ammonium ion that are normally analyzed with field samples to make corrections for reagent contamination were not included in this study, but comparable adjustments can be made here by making a correction based on the average difference between the calculated and experimental values (see Table II). Nitrogen contamination during sample preparation is not a problem for the HPLC method because sample filtrates are analyzed directly for the two isotopic forms of ammonium.

We have successfully applied the HPLC technique to measuring isotope ratios in preliminary isotopic-dilution experiments conducted in a small pond in Ann Arbor, MI, and in the Gulf of Mexico (unpublished data with J. Cotner and J. Cavaletto). In these experiments, measurements were replicated and/or made in time series. In both cases, isotope ratios and ammonium concentrations changed significantly over time, and it was possible to calculate remineralization and potential uptake rates from the HPLC data.

In conclusion, both ammonium ion concentrations and isotope composition can be estimated by HPLC from injections of small (50-μL) seawater samples (total sample size = 0.4 mL) in isotope dilution experiments. Although not suited for experiments with tracer additions of $^{15}NH_4$, the HPLC method is attractive for examining ammonium ion regeneration and potential uptake rates in aquatic ecosystems. Advantages over other available methods include direct water sample injection, potential field portability, small sample size, simultaneous determination of ammonium ion concentrations and isotope ratios, absence of contamination from atmospheric ammonia or N_2 or from reagents during sample processing, and the relatively low cost for HPLC equipment as compared to that for mass or emission spectrometers. Water samples can be filtered and injected for analysis within minutes after removing them from incubation bottles. Alternatively, samples can be immediately filtered and the filtrates frozen for later analysis. The ability to analyze samples in the field during experimental incubations is desirable because field measurements would eliminate the need to process extra samples to ensure that the selected incubation intervals are in the right range for detection of isotopic changes.

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